Summary of PhD THESIS

Supramolecular complexes of bioflavonoids with biological relevance

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(SUMMARY OF THE DOCTORAL THESIS)

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SUMMARY

Flavonoids represent one of the most abundant groups of naturally occurring polyphenols in the human diet. Due to their wide range of health-promoting properties, such as antioxidant, anti-inflammatory and protective effects, flavonoid-based food supplements have gained increasing popularity in recent years among the general population. Among these compounds, icariin, icariside II and icaritin have attracted considerable scientific attention. Historically used to treat cardiovascular disease, osteoporosis, and sexual dysfunction, icariside II has recently shown a promising pharmacological profile in several commonly encountered health disorders. However, despite its therapeutic potential, the bioavailability of these compounds remains low. For icariside II in particular, this limitation can be attributed to its poor water solubility and low membrane permeability. To address these challenges, the present study investigates supramolecular complex formation as a strategy to enhance solubility and, consequently, the bioavailability of icariside II.

Among the potential carriers suitable for complex formation, whey proteins demonstrate strong potential for several reasons. Whey proteins have many benefits to human health such as anti-carcinogenic, antimicrobial, blood pressure-lowering, appetite-suppressing, cholesterol-lowering, and tooth decay-preventing properties. Whey protein concentrate is primarily composed of β-lactoglobulin (β-lg, 50% w/w), α-lactoglobulin (20% w/w), and smaller amounts of other proteins, such as immunoglobulins and bovine serum albumin.

β-Lactoglobulin qualifies WPC as an ideal candidate for complexation with icariside II due to its ability to bind small hydrophobic molecules within its hydrophobic cavity. Additionally, whey proteins exhibit desirable functional properties including high solubility, emulsifying capacity, gel and foam formation, and water-binding ability. The main driving forces of complex formation include hydrogen bonding as well as hydrophobic and electrostatic interactions. Importantly, whey proteins are widely available on an industrial scale as a major by-product of cheese manufacturing, offering low economic cost and sustainability potential. Taken together, these characteristics make WPC a promising carrier for encapsulating bioactive molecules, including icariside II.

Spectroscopic analyses combined with differential scanning calorimetry (DSC) and X-ray powder diffraction (XRD), were employed to investigate the formation and stability of the ICS–WPC complexes. Spectrofluorimetric measurements were used to evaluate the thermodynamic behavior of the interaction, while Fourier-transform infrared spectroscopy, DSC, and XRD provided structural evidence confirming the formation of new amorphous complexes. These analyses demonstrated that complexation between icariside II and WPC is thermodynamically favorable. The impact

of surfactants, such as Tween 80 and lecithin, on aqueous solubility was also examined. Although complexation with WPC alone substantially improved solubility, the addition of surfactants led to further enhancement by promoting dispersion and reducing molecular aggregation. These findings indicate that surfactants are necessary to maximize the aqueous solubility and bioavailability, complementing the effects of the complex formation.

In addition to improving solubility, the antioxidant activity of the complexes was also evaluated. Results obtained using the DPPH· radical-scavenging assay and the Briggs–Rauscher oscillatory reaction demonstrated that complexation significantly improved the antioxidant activity of icariside II. This suggests that carrier-based modification not only improves physicochemical properties but may also strengthen biological activity.

Overall, this research introduces a biotechnology-driven strategy for enhancing the solubility of icariside II through whey protein complexation, further reinforced by the incorporation of Tween 80 and lecithin. The findings contribute to the broader field of bioactive delivery systems by improving the functionality of poorly soluble natural compounds. Additionally, by repurposing the whey proteins, a by-product of the dairy industry, the study supports sustainability goals and aligns with green processing concepts. The outcomes may be extended to other hydrophobic flavonoids, paving the way for the development of more efficient, sustainable and functionally enhanced supplement formulations within the biotechnological and nutraceutical sectors.

The main objectives of this PhD thesis

The central hypothesis of this Ph.D. project was that icariside II can be efficiently solubilized through supramolecular complex formation with whey protein concentrate, and that this effect can be further amplified by the incorporation of surfactants. This method is expected to achieve improved water solubility and antioxidant capacity, thereby facilitating its successful incorporation into food supplements. In addition, the use of whey protein, a by-product of cheese production, contributes to sustainability by repurposing resources.

To validate this hypothesis, several specific objectives were defined to guide the research and ensure a comprehensive evaluation of the proposed solubility enhancement strategy. These objectives are:

O1. Formulate and optimize icariside II and whey protein concentrate (ICS–WPC) complexes using whey protein concentrate as a natural, low-cost, and sustainable carrier, with a focus on complexation efficiency and physicochemical stability.

O2. Investigate the interactions between icariside II and WPC under various conditions (pH, temperature), using spectrofluorimetric measurements to evaluate the thermodynamic parameters and binding mechanisms.

O3. Characterize the structural transformations and stability of the ICS-WPC complexes using FTIR, XRD, DSC and molecular docking simulations, confirming the formation of new amorphous supramolecular assemblies.

O4. Investigate the effect of surfactants (Tween 80 and lecithin) on ICS-WPC complexes, and to determine the optimal ratio that ensures the greatest increase in solubility.

O5. Evaluate the antioxidant activity of the complexes using DPPH· and Briggs–Rauscher assays, determining whether complexation and surfactant incorporation improve the functional bioactivity of ICS.

This Ph.D. thesis explored novel approaches to enhance the water solubility and antioxidant activity of icariside II. Through the formation of complexes with whey protein concentrate and the incorporation of various surfactants, such as Tween 80 and lecithin, the study aimed to develop a sustainable and cost-effective food supplement.

General conclusions

The principal findings presented in this thesis are summarized as follows:

Chapter 3 investigated the possibility of forming new supramolecular complex formation between icariside II and whey protein concentrate. X-ray powder diffraction, differential scanning calorimetry, and Fourier transform infrared spectroscopy provided clear evidence of the newly formed amorphous ICS-WPC complexes. The thermodynamic analysis revealed that the most favorable conditions for the complex formation were pH 8 and 40°C. The results obtained from fluorescence spectroscopy indicated positive values of enthalpy and entropy change, highlighting that hydrophobic interactions represent the dominant driving force behind complex formation.

Chapter 4 explored the enhancement of icariside II water solubility through complexation with whey protein concentrate and through the addition of surfactants such as Tween 80 and lecithin. The study demonstrated that complexation with WPC alone significantly improved ICS solubility by approximately 258-fold. When combined with the selected surfactants, solubility was further enhanced up to 554-fold, highlighting a synergistic effect between protein-based carriers and surfactants. Spectroscopic and thermal analyses confirmed the successful formation of new complexes, while scanning electron microscopy revealed notable morphological changes. In addition, this approach demonstrated both high efficiency and sustainability by valorizing whey, a by-product of the dairy industry.

Chapter 5 investigated how the addition of surfactants influences the antioxidant activity of icariside II using BR and DPPH· assays. Results indicated that ICS exhibited minimal activity in aqueous environments; however, its antioxidant capacity was enhanced through complexation with whey protein concentrate and surfactants (Tween 80 and lecithin), resulting in a 4.16-fold increase. These results demonstrate the

effectiveness of the complexation procedure in enhancing the functionality of icariside II.

Based on the findings of this research, the following general conclusions and recommendations have been formulated:

1. The results confirmed that appropriate processing conditions enable efficient complex formation between icariside II and whey protein concentrate, yielding stable amorphous complexes.
2. Thermodynamic analyses confirmed that hydrophobic interactions were the primary driving force behind supramolecular complex formation.
3. The results demonstrate that whey protein-based complexation, particularly when combined with surfactants, provides a powerful and reliable method for improving the solubility and functional performance of icariside II. This improvement can be attributed to morphological changes and a reduction in interfacial tension.
4. Structural and morphological investigations (DSC, XRD, FTIR, SEM) confirmed successful complex formation, the transition from crystalline to amorphous state, and the formation of new hydrogen bonds. These transformations contribute to improved solubility and stability of icariside II. The valorization of whey protein, a by-product of cheese production, together with the use of spray drying, highlights the sustainability and scalability of the method with relevant implications for biotechnology.
5. Complexation of icariside II with whey protein concentrate significantly enhanced its antioxidant activity. The addition of surfactants like Tween 80 and lecithin resulted in a 4.16-fold enhancement, as confirmed by DPPH· assay.
6. The BR method revealed that pure ICS exhibited restricted bioactivity in aqueous conditions, emphasizing the importance of biotechnological formulation strategies to improve water solubility and functional performance for potential food supplement applications.
7. The developed S-ICS-WPC formulation offers a novel, sustainable, and scalable method for enhancing the bioavailability of the poorly water-soluble icariside II compound. However, further studies, such as in vitro simulated gastrointestinal and in vivo bioavailability assessments, are necessary to validate its efficacy as a functional food supplement.

Originality and personal contributions

This Ph.D. thesis presents original contributions to the field of biotechnologies and green technologies applied to functional food development, with a focus on enhancing the water solubility and antioxidant activity of poorly water-soluble natural compounds, specifically icariside II. The novelty of this research is the formation of previously unstudied complexes, introducing an innovative strategy for improving the functional properties of poorly soluble natural compounds.

One of the primary contributions of this research is the development of biotechnological delivery system based on complexation of icariside II with whey protein concentrate. To the best of current knowledge, this is the first study investigating the formation of complexes between icariside II and whey protein concentrate. Comprehensive physicochemical characterization using DSC, XRD, FTIR confirmed the successful formation of the complexes, providing solid foundation for future development of advanced food products.

The originality of the contributions is further strengthened by the incorporation of surfactants such as Tween 80 and lecithin into the ICS–WPC system, leading to the formation of a mixed surfactant-based complex (S-ICS-WPC). The addition of surfactants generated a synergistic effect, significantly amplifying the water solubility of icariside II. This study successfully demonstrated that this biotechnological approach increased solubility in aqueous media, offering a promising solution for improving bioavailability and functional effectiveness as a food supplement.

This research is among the first to evaluate the antioxidant properties of icariside II–WPC complexes using the Briggs-Rauscher oscillating system and the DPPH· radical scavenging assay. These methods, relevant to applied biotechnologies, provided both quantitative and qualitative insights under conditions that mimic the human gastric environment.

A key aspect of this research is its commitment to sustainable biotechnology, demonstrated through the use of whey protein concentrate, a valorized byproduct of the dairy industry, and the selection of spray drying as a formulation method. This modern biotechnological technique was chosen due to its affordability, accessibility, and industrial scalability, contributing to waste reduction and promoting environmentally responsible production practices.

In view of future developments, this work lays the foundation for further investigations into in vitro and in vivo bioavailability, encapsulation efficiency, and gastrointestinal stability of the developed systems. It also opens the possibility for applying similar strategies to other hydrophobic compounds in the food or pharmaceutic fields.